AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology

Establishment of Bacterial Herbicide Degraders in a Rapid Sand Filter for Bioremediation of Phenoxypropionate-Polluted Groundwater

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In this study, we investigated the establishment of natural bacterial degraders in a sand filter treating groundwater contaminated with the phenoxypropionate herbicides (*RS*)-2-(4-chloro-2-methylphenoxy)propanoic acid (MCPP) and (*RS*)-2-(2,4-dichlorophenoxy)propanoic acid (DCPP) and the associated impurity/catabolite 4-chlorophenoxypropanoic acid (4-CPP). A pilot facility was set up in a contaminated landfill site. Anaerobic groundwater was pumped up and passed through an aeration basin and subsequently through a rapid sand filter, which is characterized by a short residence time of the water in the filter. For 3 months, the degradation of DCPP, MCPP, and 4-CPP in the sand filter increased to 15 to 30% of the inlet concentration. A significant selection for natural bacterial herbicide degraders also occurred in the sand filter. Using a most-probable-number (MPN) method, we found a steady increase in the number of culturable phenoxypropionate degraders, reaching approximately 5 × 10^5 degraders per g sand by the end of the study. Using a quantitative PCR targeting the two phenoxypropionate degradation genes, *rdpA* and *sdpA*, encoding stereospecific dioxygenases, a parallel increase was observed, but with the gene copy numbers being about 2 to 3 log units higher than the MPN. In general, the *sdpA* gene was more abundant than the *rdpA* gene, and the establishment of a significant population of bacteria harboring *sdpA* occurred faster than the establishment of an *rdpA* gene-carrying population. The identities of the specific herbicide degraders in the sand filter were assessed by Illumina MiSeq sequencing of 16S rRNA genes from sand filter samples and from selected MPN plate wells. We propose a list of potential degrader bacteria involved in herbicide degradation, including representatives belonging to the *Comamonadaceae* and *Sphingomonadales*.

Groundwater is a valuable resource that is used for drinking water in many countries all over the world. The quality of the water is vital to our health, and regulations have been made to control the concentration of contaminants and protect the groundwater (1). A major concern is leaching of pesticides mainly from agricultural areas but also from urban areas or from point sources (2). In the European Union, a legal threshold limit has been set at 0.1 μg/liter for any given pesticide or 0.5 μg/liter for a total concentration of mixed pesticides in drinking water (3).

In Denmark, monitoring of the groundwater status has detected pesticides in almost 40% of the investigated wells, and the concentrations exceed the allowed threshold limit in 12% of the wells (4). Phenoxypropionate herbicides, including the compounds (*RS*)-2-(2,4-dichlorophenoxy)propanoic acid (known as DCPP or dichlorprop) and (*RS*)-2-(4-chloro-2-methylphenoxy)propanoic acid (known as MCPP or mecoprop), are among the pesticides frequently found in contaminated wells (4). The herbicides are, in particular, a problem in old landfill areas, from where they are leaching into the groundwater as a point source contamination (5). Phenoxypropionates are chemical enantiomers that exist as both *R* and *S* enantiomers. Different bacterial enzymes, namely, the (*R*)- and (*S*)-dichlorprop α-ketoglutarate dioxygenases, encoded by the *rdpA* and *sdpA* genes, respectively, are responsible for the first step in their degradation (6). Under aerobic conditions, the herbicides are subject to rapid biodegradation, and half-lives in topsoils are usually measured in days or weeks (7, 8). However, in subsoil or in groundwater aquifers with low or no oxygen availability, natural attenuation is generally very slow or absent (9). Once they are in the subsurface environment, the herbicides are, hence, very persistent, and continuous dispersal and contamination of larger groundwater reservoirs are a risk.

Polluted groundwater aquifers used for drinking water are often remediated by use of the pump-and-treat technology, where the water is pumped up and treated at the site, typically by adsorption of the contaminants onto activated carbon (10), though phenoxypropionate herbicides are not sequestered very well by activated carbon due to their chemical characteristics. As an alternative, pesticide-contaminated water may be remediated by passing the water through a sand filter with degrading bacteria. Recently, we observed the potential for the remediation of groundwater polluted by the pesticide residue 2,6-dichlorobenzamide (BAM) in a sand filter bioaugmented with specific BAM-degrading bacteria (11). Laboratory-scale sand filters with sand and bacteria from a full-scale waterworks similarly removed various organic chemicals, including the pesticides 2,4-dichlorophenoxyacetic acid (2,4-D), aldicarb, chlorpyrifos, and diazinon (12).

In an aerobic aquifer, significant *in situ* biodegradation of phenoxypropionate herbicides has previously been shown (13), and in microcosms of groundwater and sediment samples, significant degradation of the herbicides has also been observed with aerobic incubation (14–16). Similarly, in a Danish waterworks, trace concentrations of MCPP were removed from contaminated ground-

Received 10 August 2015 **Accepted** 16 November 2015

Accepted manuscript posted online 20 November 2015

Citation Feld L, Nielsen TK, Hansen LH, Aamand J, Albers CN. 2016. Establishment of bacterial herbicide degraders in a rapid sand filter for bioremediation of phenoxypropionate-polluted groundwater. Appl Environ Microbiol 82:878–887. doi:10.1128/AEM.02600-15.

Editor: F. E. Löffler

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water by filtering through a sand filter (17). These studies show that phenoxypropionate degrader bacteria are present to some extent in the groundwater environment and under appropriate conditions may be enriched to present a significant degradation potential. This supports the idea that establishment of natural degrader populations in sand filters can be used for treatment of groundwater contaminated by phenoxypropionate herbicides. However, the potential for the development and use of such a treatment strategy has, to our knowledge, been largely unexplored.

Waterworks sand filters harbor diverse populations of bacteria shaped by the inlet water chemistry (18), but very little is known about the successive development of such microbial communities, including the establishment of pesticide-degrading populations. The aim of this study was to investigate the natural potential of specific phenoxypropionate herbicide degraders for biodegradation of the herbicides MCPP and DCPP and the associated impurity/catabolite 4-chlorophenoxypropanoic acid (4-CPP) in a sand filter for remediation of polluted groundwater. The main objective was to explore the abundance and identity of specific degraders and to obtain a better understanding of the colonization by these bacteria following the start-up of a pump-and-treat sand filter facility.

MATERIALS AND METHODS

Groundwater characteristics of field site. A pilot facility was installed outside Copenhagen, Denmark (55°36′34″N, 12°09′14″E), in an area previously used to dig for gravel and later as a landfill. The groundwater in this area is subject to widespread pollution with the phenoxypropionate herbicides MCPP and DCPP as well as 4-CPP. 4-CPP is known to occur as an impurity in the production of both DCPP and MCPP, and the presence of this compound may thus derive directly from the original discharge of the herbicides (19). In addition, it has been proposed that reductive dechlorination of DCPP under anaerobic conditions can lead to the formation of 4-CPP (20). This process may potentially occur in the groundwater reservoir, but under the aerobic conditions in the sand filter, the biodegradation of DCPP does not lead to the production of 4-CPP.

The concentrations of the herbicides in the inlet groundwater were measured during the experiment, and the concentrations were 1.0 to 1.2 μg/liter for MCPP, 0.14 to 0.17 μg/liter for DCPP, and 0.66 to 1.0 μg/liter for 4-CPP. Other general chemical characteristics of the inlet groundwater were measured as previously described (11); total iron was present at 2 mg/liter, total manganese at 0.1 mg/liter, ammonium at 0.3 mg/liter, methane at 0.6 mg/liter, nonvolatile organic carbon at 10 mg/liter, chloride at 155 mg/liter, and sulfate at 103 mg/liter, and the pH was 6.8. The concentration of total P was 0.005 mg/liter, which was measured using the ISO 6878:2004 standard at a commercial laboratory (ALS Denmark). The temperature of the abstracted groundwater varied from 10.5 to 11.0°C throughout the study, and during filtration the temperature in the filters remained relatively constant.

Pilot facility. The pilot facility consisted of a container equipped with pumps and an aeration basin followed by a rapid sand filter, i.e., a filter operated with a fast water flow, which is necessary for remediation of large water volumes. Rapid sand filters typically have residence times of from a few minutes to 20 min and use coarse sand materials (particle size, >0.8 mm), while slow sand filters have residence times far longer than 1 h and use fine sand materials (particle size, <0.5 mm). The sand filter was assembled in a Plexiglas cylinder with a lid and was operated with a downward flow, as described in Fig. 1. Water sampling was managed through ports placed before the aeration basin (raw water), between the aeration basin and the sand filter (inlet water), and after the sand filter (outlet).

A 69-day preexperiment was run prior to the study, to test the aeration and flow conditions of the system. In the preexperiment, full oxygen saturation of the raw water was carried out in the aeration basin, giving an oxygen concentration of 11 mg/liter in the inlet water, as determined with an optical oxygen sensor (SC-FDO; WTW, Weilheim, Germany). However, despite frequent (daily) backwashing of the sand filter, a high level of precipitation of calcium carbonates resulted in cementation and blocking of the filter. Backwashing is a normal procedure carried out at waterworks to prevent clogging of the filter by calcium carbonate, iron oxides, and manganese oxides.

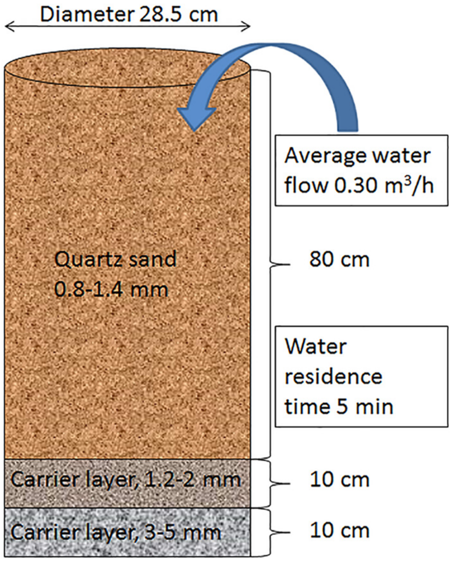

FIG 1 Characteristics of the sand filter and water flow conditions.

On the basis of the results of the preexperiment, a decreased aeration resulting in 6 mg/liter oxygen in the inlet water was chosen for the study, in order to reduce precipitates. Prior to start-up, the sand in the filter was exchanged with new filter sand. To remove precipitates from the sand filter, backwashing was carried out every 1 to 2 days by circumventing the water flow and flushing with water from the outlet of the filter.

Determination of herbicide removal. Water to be analyzed for herbicides was immediately filtered through 0.22-μm-pore-size hydrophilic polytetrafluoroethylene filters (Q-Max, Frisenette, Denmark) to avoid degradation before analysis. Beforehand, the adsorption of phenoxy acid herbicides to the filters was tested, using 1-μg/liter concentrations of ^{14}C-labeled MCPP, DCPP, and 2-methyl-4-chlorophenoxyacetic acid (MCPA). In all cases, recovery was >99%, demonstrating that filtration could be safely performed. Herbicide concentrations were determined by liquid chromatography-tandem mass spectrometry analysis by a commercial DANAK-accredited analytical laboratory (Højvang Laboratorier A/S, Dianalund, Denmark). The following phenoxy acid herbicides and derived metabolites/production impurities were analyzed: MCPP, DCPP, 2,4-D, MCPA, 4-CPP, 4-chloro-2-methylphenol, and 2,4-dichlorophenol. The detection limit for all compounds was 0.01 μg/liter, and the relative analytical uncertainty was 15% (95% confidence limit). Only MCPP, DCPP, and 4-CPP were detected at any sampling date.

Sampling of sand filter material. Filter material was sampled from the top of the sand filter using a spoon rinsed with ethanol. Samples from the middle of the filter were taken using a custom-made pipe system (11). Samples of filter material were brought to the laboratory and immediately processed for most-probable-number (MPN) analysis of phenoxypropionate degraders. Samples for DNA extraction were stored at −20°C until further use. At the last sampling day of the experiment, filter samples were also collected for RNA analysis. Duplicate samples of approximately 0.5 g (dry weight) filter sand were transferred to 2-ml PCR tubes (SC microtube PCR-PT; Sarstedt, Germany) and frozen on-site in liquid nitrogen. The samples were then brought to the laboratory and stored at −80°C until analysis.

Quantification of phenoxypropionate herbicide degraders by most probable number. The number of bacteria capable of phenoxypropionate degradation in the sand filter was determined by MPN, using a radiorespirometric method (21) that was modified to quantify phenoxypropionate degraders. In short, a slurry of the filter samples was prepared from 1 g of sand and 9 ml of half-strength Bushnell-Hass broth (Becton, Dickinson and Company, Sparks, MD, USA), which was mixed by end-to-end shaking at 200 rpm for 10 min and thereafter left to settle for 10 min. The slurry was subsequently used to prepare 3-fold dilutions in half-strength Bushnell-Hass broth. To a 96-well microplate, 200 μl of each dilution was added in five replicates, and each well was then added to 10 μl of an aqueous phenoxypropionate solution (a 400-mg/liter nonlabeled DCPP solution and a 120,000-dpm/ml ^{14}C-ring-labeled DCPP solution) as the sole carbon source for growth. The plates were sealed with microplate sealing tape with $Ca(OH)_2$ filter traps and incubated at room temperature. The radioactivity collected in the sealing tapes was checked after 1, 3, and 5 weeks, as previously described (21). The MPN of phenoxypropionate degraders was calculated from these observations using the freeware program MPNAssay analyzer (Special K Software Group).

Extraction of DNA and RNA. Coextraction of DNA and RNA from freeze-dried samples was done using a PowerMicrobiome RNA isolation kit (catalog no. 26000-50; MO BIO Laboratories, Inc., Carlsbad, CA, USA) with a modified procedure as previously described (11). The extracted RNA was immediately converted to cDNA using, first, an RTS DNase kit (MO BIO Laboratories) and, subsequently, a RevertAid Premium RT kit in combination with random hexamer primers (Thermo Scientific, Inc., Lithuania) for reverse transcription-PCR, according to the manufacturers' instructions. The DNA and cDNA samples were stored at −20°C until they were used for quantitative PCR (qPCR) analysis and sequencing library preparation. A subsample of the RNA was retrieved after DNase treatment and was stored at −80°C to be used as a negative control to ensure the absence of contaminating DNA in the cDNA samples.

An inhibition test was performed on the extracted DNA and cDNA samples to ensure the absence of PCR inhibitors. Determination of quantification cycle (C_q) values was used to test inhibition, when a specific plasmid DNA template was amplified in qPCRs with and without addition of the DNA or cDNA sample. A plasmid derived from the *Escherichia coli* pCR 2.1-TOPO vector and primers M13-forward and M13-reverse, targeting a specific region in the vector, were used (Invitrogen, Carlsbad, CA). PCR mixtures were prepared with 1× SsoFast EvaGreen supermix (Bio-Rad), 1 mg/ml bovine serum albumin (BSA; Bioron), 1 μl plasmid template, primers M13-f and M13-r (0.4 μM each), and 1 μl of either the DNA/cDNA to be tested for inhibition or PCR-grade water (no-inhibition control). An increase in the C_q value compared to that for the no-inhibition controls was indicative of the presence of PCR inhibitors in the extracted DNA/cDNA. In some samples, particularly those from the tops of the filters, inhibition was observed, possibly due to coextracted iron oxides. Samples showing inhibition were diluted 10- to 50-fold, and the samples were then checked again until no inhibition was observed, before being used as the templates in the qPCR assays.

Quantification of phenoxypropionate degrader genes by qPCR. The numbers of *rdpA* and *sdpA* phenoxypropionate degrader genes and mRNA transcripts were quantified by qPCR using a CFX96 real-time system (Bio-Rad Laboratories, USA) or an iCycler thermocycler (Bio-Rad). PCR mixtures were prepared with 1× SsoFast EvaGreen supermix (Bio-Rad), 1 mg/ml BSA (Bioron), 1 μl DNA or cDNA template, and primers rdpA_f and rdpA_r (0.4 μM each for the *rdpA* assay) or sdpA_f (0.4 μM) and a mixture of sdpA_r1 and sdpA_r2 (0.2 μM each for the *sdpA* assay), as previously described (22). PCR cycling conditions were as follows: 98°C for 2 min, 40 cycles of 98°C for 10 s and 55°C for 20 s (CFX96) or 30 s (iCycler), and 72°C for 2 min. In the end, a dissociation curve of the amplification products was prepared by increasing the temperature from 60°C to 95°C in increments of 0.1°C (CFX96) or 0.5°C (iCycler).

Delftia acidovorans MC1 carrying the *rdpA* and *sdpA* genes was used as a standard (23, 24). The strain was grown under selective conditions in mineral salts solution supplemented with 400 mg/liter (*R*/*S*)-DCPP, as previously described (22). Duplicate 10-fold dilutions of the *D. acidovorans* MC1 culture were prepared in a 0.9% NaCl solution, and 100 μl of each dilution was spiked into tubes with 500 mg fresh sand (like that used to prepare the sand filters). The spiked samples were flash-frozen in liquid nitrogen and freeze-dried before extraction of DNA, as described above. The duplicates from each extraction were then mixed and used to prepare a standard curve for qPCR quantification of the number of *rdpA* and *sdpA* copies in the sand filter. By spiking of the MC1 strain to the specific sand matrix, the standard curves accounted for both the efficiency of DNA extraction from the sand and the PCR amplification efficiency. For the *rdpA* and *sdpA* assay, standard values used for quantification were obtained within the range of 5×10^2 to 5×10^5 *rdpA* or *sdpA* templates per PCR mixture (corresponding to 5×10^4 to 5×10^7 *rdpA* or *sdpA* copies g^{-1} sand). Parameters for *rdpA* were a qPCR efficiency (E) of 61.5%, R^2 of 0.93, a slope of −4.82, and a y intercept of 47.1. Parameters for *sdpA* were an E of 54.4, R^2 of 0.94, a slope of −5.31, and a y intercept of 54.1.

The standards for the qPCR assays were prepared by extraction of DNA from appropriate bacterial cultures. The efficiency of reverse transcription of RNA to cDNA and a potential loss of mRNA by degradation during the extraction procedure were, hence, not accounted for in the quantification of mRNA transcript numbers. This may have resulted in a minor underestimation of the number of mRNA transcripts actually present in the sand filters.

Quantification of total bacteria in the sand filter. Total bacterial numbers in the sand filter were quantified by qPCR targeting the 16S rRNA gene. qPCR was carried out using 1× SsoFast EvaGreen supermix (Bio-Rad), 0.4 μM (each) forward primer 341F (5′-CCTACGGGAGGCAGCAG-3′) and reverse primer 518R (5′-ATTACCGCGGCTGCTGG-3′) (25), 1 mg/ml BSA (Bioron), and 1 μl DNA template. Amplification was carried out in a CFX96 real-time system with PCR cycling at 98°C for 2 min followed by 40 cycles of 98°C for 10 s and 55°C for 20 s. Finally, an elongation step at 72°C for 2 min and a melt curve analysis were performed. *Escherichia coli* K-12 with seven 16S rRNA gene copies per genome was used as a standard (26), and the quantification parameters were an E of 96.2%, R^2 of 0.99, a slope of −3.42, and a y intercept of 38.6. An assumption of 4 copies per genome in sand filter bacteria was made to calculate the number of bacteria (27).

Library preparation for investigation of bacterial 16S rRNA diversity. A library was prepared to investigate the diversity of 16S rRNA genes in the sand filter from days 14, 57, and 85 and in cDNA from day 85. To investigate the identity of specific phenoxypropionate degraders, DNA from the selective MPN plates from day 14 was also included in the library (samples from 30-fold-diluted wells in technical triplicates).

Initially, amplification of the 16S rRNA gene and cDNA was carried out in a Bio-Rad S1000 thermal cycler with PCR cycling at 95°C for 2 min, followed by 35 cycles of 95°C for 15 s, 55°C for 15 s, and 68°C for 40 s and, finally, an elongation step at 68°C for 4 min. The PCR mixtures contained 1× AccuPrime SuperMix II (Invitrogen, Eugene, OR, USA), 10 μM (each) forward and reverse primers, 7.5 to 75 ng DNA or cDNA template (in some samples, less template was available), and DNase/RNase-free water to a final volume of 30 μl. The primer pair selected for sequencing of the V3-V4 variable region of 16S rRNA genes was 341f (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) (28), and Illumina Nextera overhang adapters were attached to the 5′ ends of the primers (adapter on forward primer, 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′; adapter on reverse primer, 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′; Illumina Inc., San Diego, CA, USA). Prior to PCR amplification, the reaction mixtures were split into three tubes of 10 μl each, to reduce potential PCR amplification biases (29). Following amplification, the contents of the three tubes were mixed again to obtain one sample. The PCR products were checked for the correct size on a 1% agarose gel before being used as the template in the second PCR. In the second PCR, index sequences and sequencing adapters were added to the first PCR products using a Nextera XT index kit (Illumina Inc.). Twelve microliters of AccuPrime SuperMix II (Invitrogen), 2 μl of each primer (Illumina Inc.), 7 μl PCR-grade water, and 2 μl product from the first PCR were mixed for the second PCR, resulting in a final volume of 25 μl, which was run for 1 min of denaturation at 98°C followed by 13 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 20 s, and elongation at 68°C for 20 s. PCR cycling was followed by a final elongation step at 68°C for 5 min.

PCR products were purified using HighPrep PCR magnetic beads, according to the manufacturer's instructions (Magbio Genomics, Gaithersburg, MD, USA) and eluted in 27 μl resuspension buffer (Illumina Inc.). DNA concentrations were measured on a Qubit (version 2.0) fluorometer (Invitrogen, Eugene, OR, USA), and purified products were pooled in equimolar concentrations for sequencing on an Illumina MiSeq sequencer, using a 250-bp-read-length paired-end protocol.

Sequencing and bioinformatics. Using the USEARCH tool (version 7.0.1090) (30), demultiplexed paired-end reads were merged and length filtered, allowing only merged reads longer than 400 bp and an expected error frequency of 0.5 per read. After filtering, the primers used in the PCR were identified in the reads and removed. In the UPARSE pipeline (31), quality filtered reads were dereplicated, filtered for singletons, clustered, and filtered for chimeras by comparison with the sequences in the RDP Gold database (version 9) (32). The reads were mapped back to chimera-filtered clusters with the USEARCH tool at a 97% identity cutoff, corresponding to the genus level.

In QIIME (version 1.8.0) (33), operational taxonomic units (OTUs) making up less than 1% of the total abundance were assembled into a category referred to as "other," since the aim was to identify changes in the abundances of key bacterial species and not to focus on rare OTUs. Twenty-five OTUs had a total abundance higher than 1%, while 469 OTUs constituted the rare OTUs that represented the category other. Subsequently, samples were rarefied to contain the same number of reads ($n =$ 5,288), and diversity estimates (the Chao1 richness and Shannon-Weaver diversity indices) were calculated. Taxonomy was assigned to the reads, using the LCAClassifier taxonomic classifier (34), by comparison with the sequences in the Greengenes database (version 13_8) (35), and OTU tables were made with the biological observation matrix (BIOM) format (36) and imported into SigmaPlot software (version 12.0; Systat Software, Inc., CA, USA) to make plots and perform statistical analyses on relative OTU abundances. Where possible, OTUs were assigned to the genus level; however, some OTUs could not be determined to a level lower than class, order, or family. In order to determine which OTUs were selected for in the MPN assay, one-way analyses of variance were performed to compare the sand filter DNA samples with the MPN samples, using the rarefied OTU table. Results are shown as the mean ± standard deviation OTU abundance.

Sorption and coprecipitation experiments. The sorption of DCPP and MCPP to filter material with and without iron oxide precipitates was investigated by shaking 5 ml herbicide solution (0.2, 2, 20, and 200 μg/liter of ^{14}C-labeled DCPP and MCPP [Institute of Isotopes Co., Ltd., Budapest, Hungary; purity at the moment of usage, ≥97%]) with 5 g (wet weight) filter material. NaN_3 was added to a concentration of 0.5% to avoid biological degradation during incubation. The pH was kept at 7.2 by a 15 mM phosphate buffer. After 6 h of shaking, the vials were centrifuged (350 × *g*, 5 min), and 2 ml of supernatant was sampled to determine the ^{14}C activity in the water by liquid scintillation counting (Tri-Carb 2810 TR; PerkinElmer, Waltham, MA). The following filter materials were used: fresh quartz sand, a sample from the top of the filter after the end of the experiment (iron oxide coated), and no filter material (control).

Coprecipitation of the herbicides with iron oxide precipitation was investigated by adding ^{14}C-labeled DCPP and MCPP to an iron(III) chloride-quartz sand slurry and then raising the pH to 7 to allow precipitation of iron oxides on the sand grains, as previously described (37).

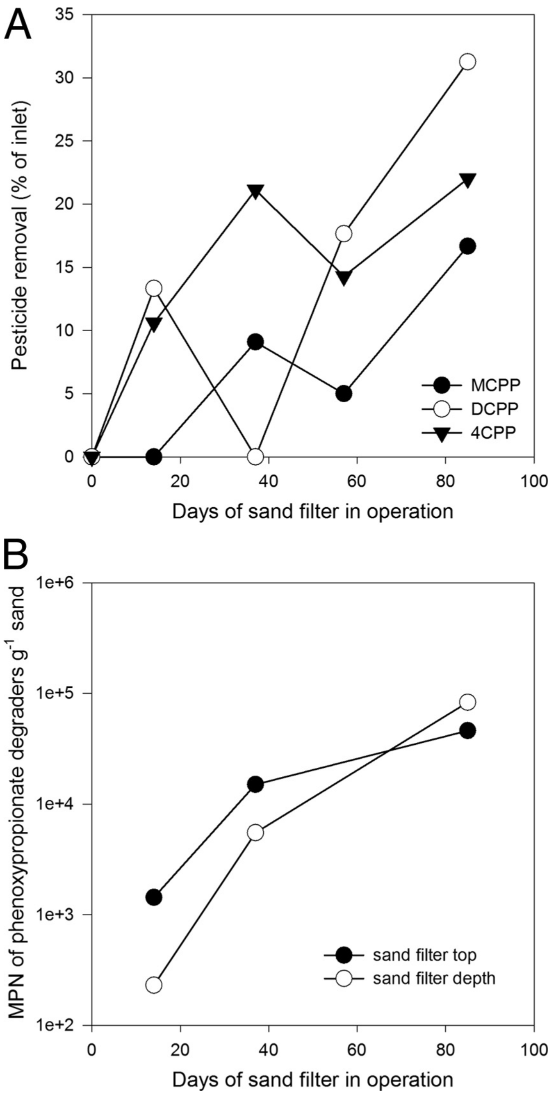

FIG 2 (A) Removal of MCPP, DCPP, and 4-CPP in the sand filter during the 85 days of the study. The removal in the sand filter is calculated as the difference in herbicide concentration between the inlet and the outlet water. (B) Development in the MPN of specific culturable phenoxypropionate herbicide degraders in the top and in the depth of the sand filter. The MPN was determined by selective culturing of diluted sand filter samples in minimal medium with DCPP as the sole C source.

Nucleotide sequence accession numbers. The sequence data have been deposited in the NCBI database under accession numbers SRR2155574, SRR2155580, and SRR2155582.

RESULTS

Degradation of phenoxypropionate herbicides in the sand filter. A significant degradation of the herbicides DCPP and MCPP and the associated compound 4-CPP was achieved by filtration through the rapid sand filter that was set up in this study. At the time of start-up of the facility, we observed no removal of the herbicides, but gradually, during the 85 days of the study, the potential for the degradation of all three compounds in the sand filter was established (Fig. 2A). A period of approximately 1.5 months was required before significant degradation of 4-CPP occurred, and degradation of DCPP and MCPP was observed after slightly longer periods of 2 and 3 months, respectively. By the end of the study, the degradation of 4-CPP, DCPP, and MCPP was approximately 22%, 31%, and 17%, respectively (Fig. 2A).

Sorption and coprecipitation. Sorption was negligible and below the lowest equilibrium coefficient (K_d) of 0.05 liter/kg that could be quantitatively determined for all filter materials and at all concentrations investigated. Coprecipitation of the herbicides with iron oxide precipitation was also not detectable; thus, abiotic factors do not seem to contribute to herbicide removal in the filter.

Culturable phenoxypropionate degraders quantified by MPN. Quantification of MPN showed that a population of phenoxypropionate degraders gradually colonized the filter sand. After 2 weeks, approximately 10^3 culturable degraders per g sand were present in the top of the filter and a slightly lower number of 2×10^2 degraders per g sand was observed in the depth of the filter. Following adaptation throughout the study, the number increased to approximately 5×10^4 and 8×10^4 degraders per g sand in the top and the depth of the filter, respectively (Fig. 2B).

Total bacteria and herbicide degradation genes in the sand filter. The total number of bacteria present in the sand filter was quantified by a qPCR targeting the 16S rRNA gene. Bacterial colonization of the fresh sand occurred rapidly after the filter was set into operation. After 14 days, a large population comprising approximately 4×10^8 bacteria per g sand had established in the top of the filter (Fig. 3A). The population subsequently increased to 1×10^9 bacteria per g sand and remained relatively stable throughout the study (Fig. 3A). Colonization was slower in the depth of the filter. After 14 days, the population size was 2×10^6 bacteria per g sand, but the number steadily increased up to 1×10^8 bacteria per g sand by the end of the study (Fig. 3A).

The *sdpA* gene, required for the first degradation step of the *S* enantiomer of phenoxypropionate herbicides, was present in high numbers of approximately 10^7 gene copies per g sand in the top of the filter. This number was already established at 14 days after the start-up of the filter, and it remained relatively constant until the end of the study (Fig. 3B). In the depth of the filter, the number of copies of the *sdpA* gene was significantly lower at less than 10^5/g sand after 14 days. However, colonization by bacteria carrying the *sdpA* gene also steadily took place in the depth of the filter (Fig. 3B). By the end of the study, a homogeneous concentration of approximately 5×10^7 *sdpA* copies per g sand was present in both the top and the depth of the filter (Fig. 3B).

Colonization of the sand filter with bacteria carrying the *rdpA* gene, needed for the first degradation step of the *R* enantiomer of phenoxypropionate herbicides, occurred much slower than the corresponding colonization with bacteria carrying the *sdpA* gene. After 57 days, the abundance of the *rdpA* gene was still below 10^5 copies per g sand (Fig. 3C). However, after 85 days, a population of bacteria had established, resulting in 10^7 *rdpA* copies per g sand in the top sand and 3×10^6 in the depth (Fig. 3C).

Activity of bacteria in the sand filter. The overall activity of the bacterial community in the sand filter was assessed by qPCR quantification of 16S rRNA from day 85. A high number of 2×10^{13} 16S rRNA copies per g sand was found in the top of the filter, and a lower number of 4×10^{11} 16S rRNA copies was found in the depth of the filter.

The activity of phenoxypropionate degraders was also assessed at day 85 by detection of *sdpA* and *rdpA* gene transcripts. Both *sdpA* and *rdpA* mRNA transcripts were detected in the system, indicating that the (*R*)- and (*S*)-dichlorprop α-ketoglutarate dioxygenases in fact were expressed in the sand filter. The *sdpA* mRNA transcripts were relatively homogeneously distributed throughout the filter, with 1×10^6 and 2×10^6 transcripts per g sand being found in the top and the depth, respectively. The number of *rdpA* mRNA transcripts in the top of the filter was 2×10^5 copies per g sand. However, deeper in the filter, the number of *rdpA* mRNA transcripts was below the detection limit of 1×10^5 transcripts per g sand.

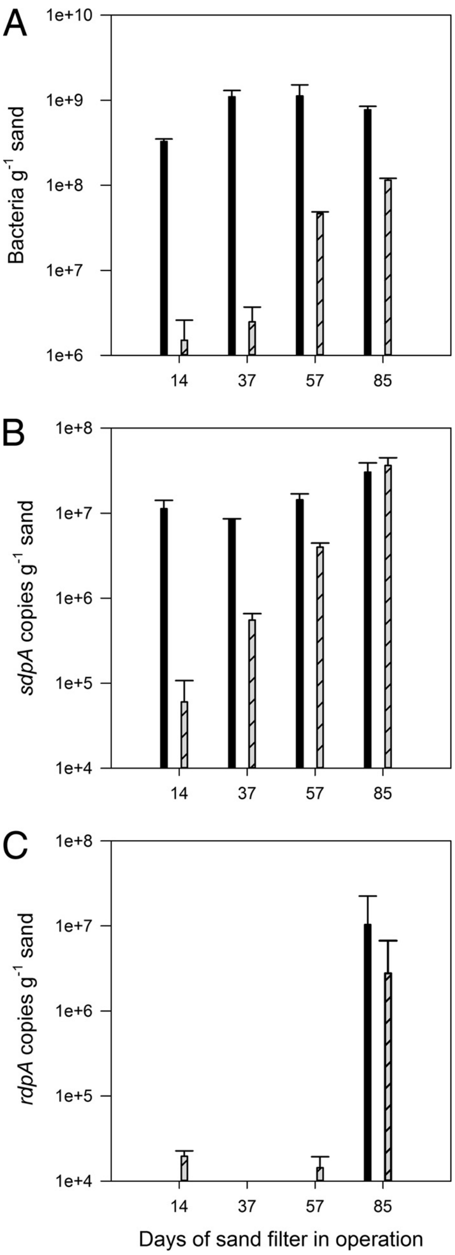

FIG 3 Development of the total number of bacteria (A), number of *sdpA* gene copies (B), and number of *rdpA* gene copies (C) in the top (black bars) and depth (hatched bars) of the sand filter. The numbers were determined by qPCR of the 16S rRNA, *sdpA*, and *rdpA* genes. The number of bacteria was estimated by assuming an average of 4.5 16S rRNA gene copies per cell. Due to the presence of PCR-inhibitory substances in the template DNA, the detection level for *sdpA* and *rdpA* was higher for the top filter samples (10^5 copies per g sand) than the depth samples (10^4 copies per g sand). The error bars show the standard deviations for triplicate DNA extractions.

TABLE 1 Chao1 and Shannon-Weaver diversity indices for 16S rRNA amplicon sequences from sand filter and MPN samples[a]

Sample	Chao1 index (SD)	Shannon-Weaver index (SD)
Day 14, top, DNA	140 (4.1)	4.0 (0.0)
Day 14, depth, DNA	137 (4.4)	3.9 (0.2)
Day 57, top, DNA	137 (1.3)	4.1 (0.0)
Day 57, depth, DNA	177 (12)	4.2 (0.1)
Day 85, top, DNA	156 (0.3)	4.0 (0.2)
Day 85, depth, DNA	190 (17)	4.5 (0.2)
Day 85, top, cDNA	80 (18)	0.7 (0.0)
Day 85, depth, cDNA	179 (5.4)	5.3 (0.0)
Day 85, top, MPN	99 (1.7)	3.3 (0.1)
Day 85, depth, MPN	39 (2.3)	2.7 (0.3)

[a] The table shows average values for duplicate samples (triplicate samples for MPN) obtained from each sample type, and standard deviations are shown in parentheses.

Bacterial diversity in the sand filter. The bacterial diversity was studied during colonization of the sand filter by amplicon sequencing of 16S rRNA genes after 14, 57, and 85 days. The diversity of the total bacterial population in the sand filter DNA samples was relatively constant from day 14 and onwards, though with a tendency toward increasing diversity in the depth of the filter (Table 1). The cDNA samples, representing the active community, showed that in the top of the filter, the diversity of the active bacteria was very low compared to that of the total bacterial population present at day 85. In contrast, the diversity of the active bacteria in the depth of the filter reflected that of the total population. For the MPN samples selective for phenoxypropionate degraders, the diversity was greatly reduced compared to that of the corresponding sand filter samples, as was also expected following selective growth. The Chao1 index was reduced from 155.7 to 99.4 for the MPN samples prepared from the top of the filter, and for the depth samples, a reduction from 190.2 to 38.8 was observed following selective growth in the MPN plates (Table 1).

A significant difference in the bacterial composition was found between the top and the depth of the filter, but the age of the sand filter also influenced the bacterial composition (Fig. 4). C_1-oxidizing bacteria, including members of the family of *Methylophilaceae* and the methanotrophic genus *Crenothrix*, were dominant members of the sand filter community and were particularly abundant in the top. Members of the genera *Sphingobium* and *Mycobacterium* also constituted a large proportion of the community, and these bacteria were particularly more abundant in the depth of the filter. Among other functional bacterial groups that dominated in the sand filter were the hydrogen-oxidizing genus *Hydrogenophaga*, the methane-oxidizing genus *Methylomonas*, and the sulfur-oxidizing genus *Sulfuricurvum* (Fig. 4).

The composition of the 16S rRNA samples, which represent the active part of the population, showed an almost total dominance of *Crenothrix* in the top of the filter (corresponding with the low diversity index), which indicates that this genus was highly active and probably also contained a high number of ribosomes per cell. In the depth samples, the composition of the actively transcribed 16S rRNA resembled the composition of 16S rRNA genes, but with a relatively greater abundance of *Methylomonas*, *Crenothrix*, and *Sphingomonadales* and a relatively lower abundance of *Mycobacterium* in the cDNA samples (Fig. 4).

During establishment of the bacterial population in the sand filter facility, we found that a number of OTUs were successful colonizers. Bacteria belonging to, e.g., *Sphingomonas*, *Sphingomonadaceae*, *Comamonadaceae*, *Xanthomonadaceae*, *Mycobacterium*, and *Crenothrix* were all enriched in the sand filter during the study (Fig. 4).

Classification of bacteria in MPN plates selective for phenoxypropionate degraders. The MPN plates enriched for phe-

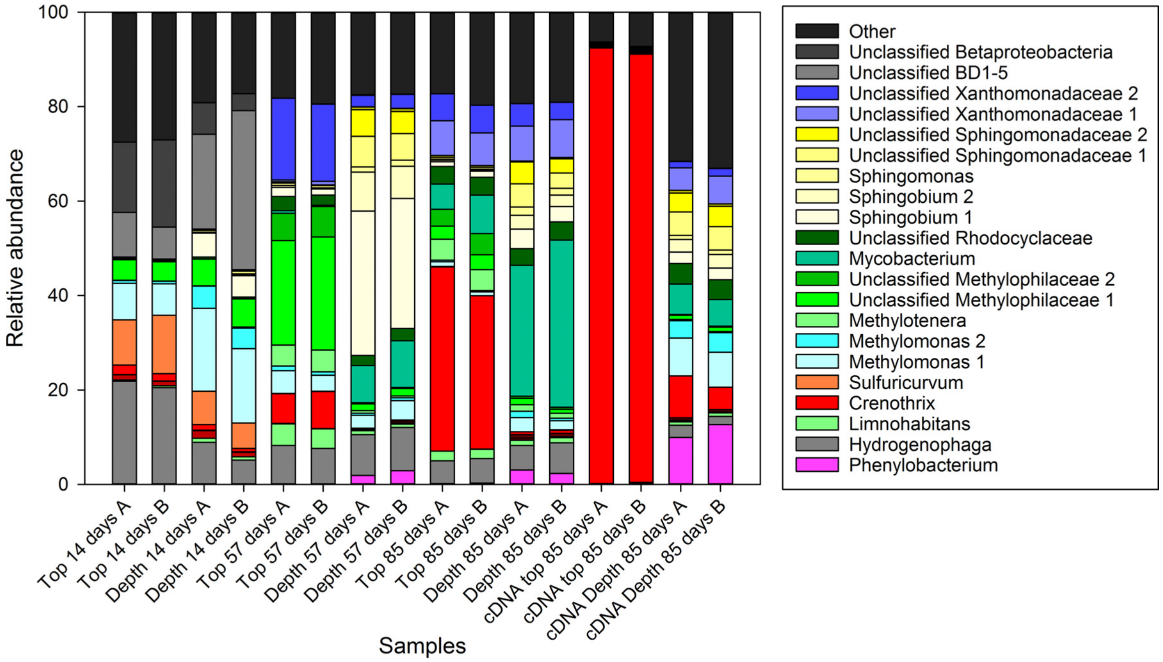

FIG 4 Composition of bacterial 16S rRNA genes in duplicate samples (samples A and B) from the sand filter collected at days 14, 57, and 85. The key shows the identities of the 16S rRNA genes that had an accumulated relative abundance of >1% across all samples.

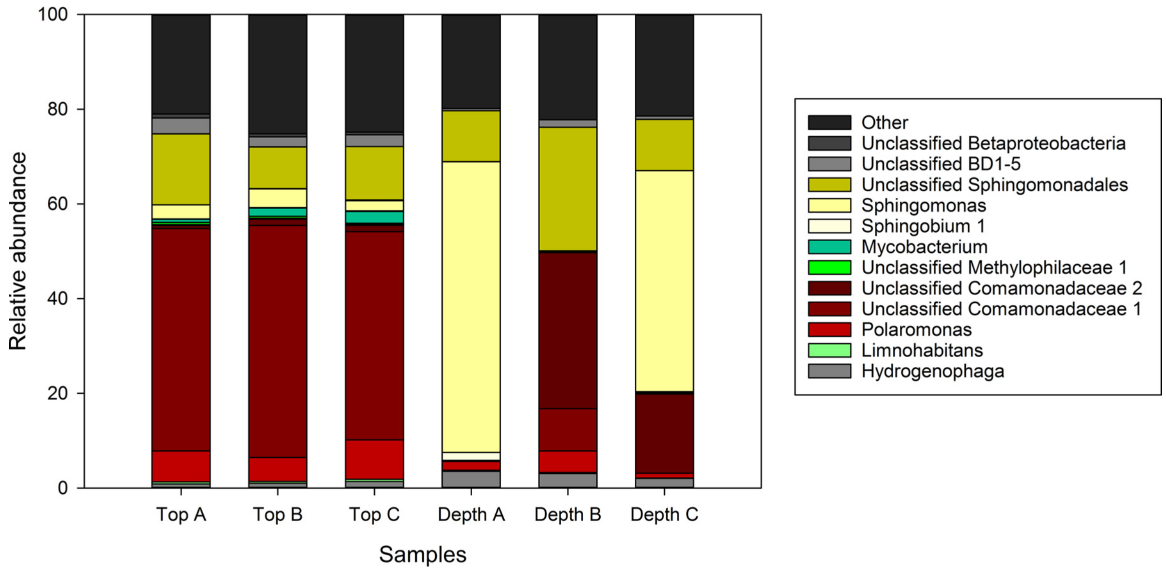

FIG 5 Composition of bacterial 16S rRNA genes in MPN samples (replicates A, B, and C) prepared from the top and depth of the sand filter at day 14. The key shows the identities of OTUs based on 16S rRNA genes that had an accumulated relative abundance of >1% across all samples.

noxypropionate degraders clearly showed a selection for specific bacteria from the sand. Altogether, the 16S rRNA reads were classified into 17 taxonomic groups, which represented 21 specific OTUs (Fig. 5).

The three MPN samples prepared from the top filter sand were highly similar in composition. In comparison, the compositions of the triplicate samples from the depth were more variable (Fig. 5). In MPN samples from the top as well as one sample from the depth, bacteria belonging to the family *Comamonadaceae* dominated and comprised approximately 56% of the population, whereas *Sphingomonas* comprised a minor fraction of approximately 3% of the samples. The opposite occurred in two of the samples from the depth, where *Sphingomonas* dominated at the expense of the *Comamonadaceae*, comprising 47 to 61% of the population. The third most abundant OTU in the MPN samples belonged to the order *Sphingomonadales* and comprised 9 to 26% of the 16S rRNA reads in all samples (Fig. 5).

Four OTUs belonging to the *Comamonadaceae* (99% similar to *Acidovorax delafieldii* [GenBank accession number NR_028714.1]), *Sphingomonadales*, *Sphingomonas*, and *Polaromonas* were significantly enriched in all MPN samples relative to their respective sand samples. Furthermore, an OTU belonging to the *Comamonadaceae* (99% similar to *Acidovorax facilis* [GenBank accession number NR_116132.1]) showed a significant enrichment in some of the MPN samples (Fig. 6).

DISCUSSION

By following a newly established rapid sand filter facility closely for 85 days, we were able to detect a continuous increase in both the total number of bacteria and the number of specific phenoxypropionate degraders. The maximum sustainable density of the total bacterial population largely seemed to have been reached within the 85 days. The bacterial density was lower in the depth than the top of the filter, presumably due to the exhaustion of easily accessible energy sources like methane and reduced iron with increasing filter depth.

A lag phase was observed before degradation of up to 30% of the phenoxypropionate herbicides occurred. This degradation was achieved at a water residence time of only 5 min, showing the potential for the use of this technique for bioremediation. Furthermore, the degrader population may still have been developing by the end of the study, and a higher level of degradation could potentially be achieved with a continued operation.

Although the specific composition of energy sources for the phenoxypropionate degraders is unknown, it seems likely that the content of phenoxypropionate herbicides in the abstracted groundwater was adequate to shape the development of the degrader population in the sand filter. Considering an average of 15% degradation during the 85 days, a total degradation of 0.2 g phenoxypropionate, or roughly 0.1 g organic carbon from the herbicides, was available for use for growth during this period. Assuming an equal distribution of degraders in the 80-cm (100-kg) filter sand, this corresponds to 1 μg carbon from phenoxypropionate per g of sand. Organic carbon typically yields 10^{12} to 10^{13} bacteria per gram (38, 39), and herbicide degradation could therefore yield 10^6 to 10^7 cells per g filter sand. At day 85, the MPN count was approximately 10^5 bacteria/g. It is thus possible that phenoxypropionate degradation was a main source of carbon for the degrader bacteria, even though maintenance of existing biomass is not included in this calculation.

The colonization by bacteria harboring the *sdpA* degradation gene was faster in the top than in the depth of the filter, similar to the findings for general bacterial colonization. However, the density of the *sdpA* gene ultimately reached the same level in the depth as in the top of the filter. When calculating the proportion between *sdpA* gene copy numbers and bacterial numbers, a ratio of 0.7 to 4% *sdpA* gene copy number to bacterial number was found for the top of the filter, whereas in the depth, the ratio increased from approximately 4% at day 14 to 28% by the end of the study. This indicates that the population of degrader bacteria was positively selected for in the depth but comprised a smaller and more constant proportion of the total population in the top.

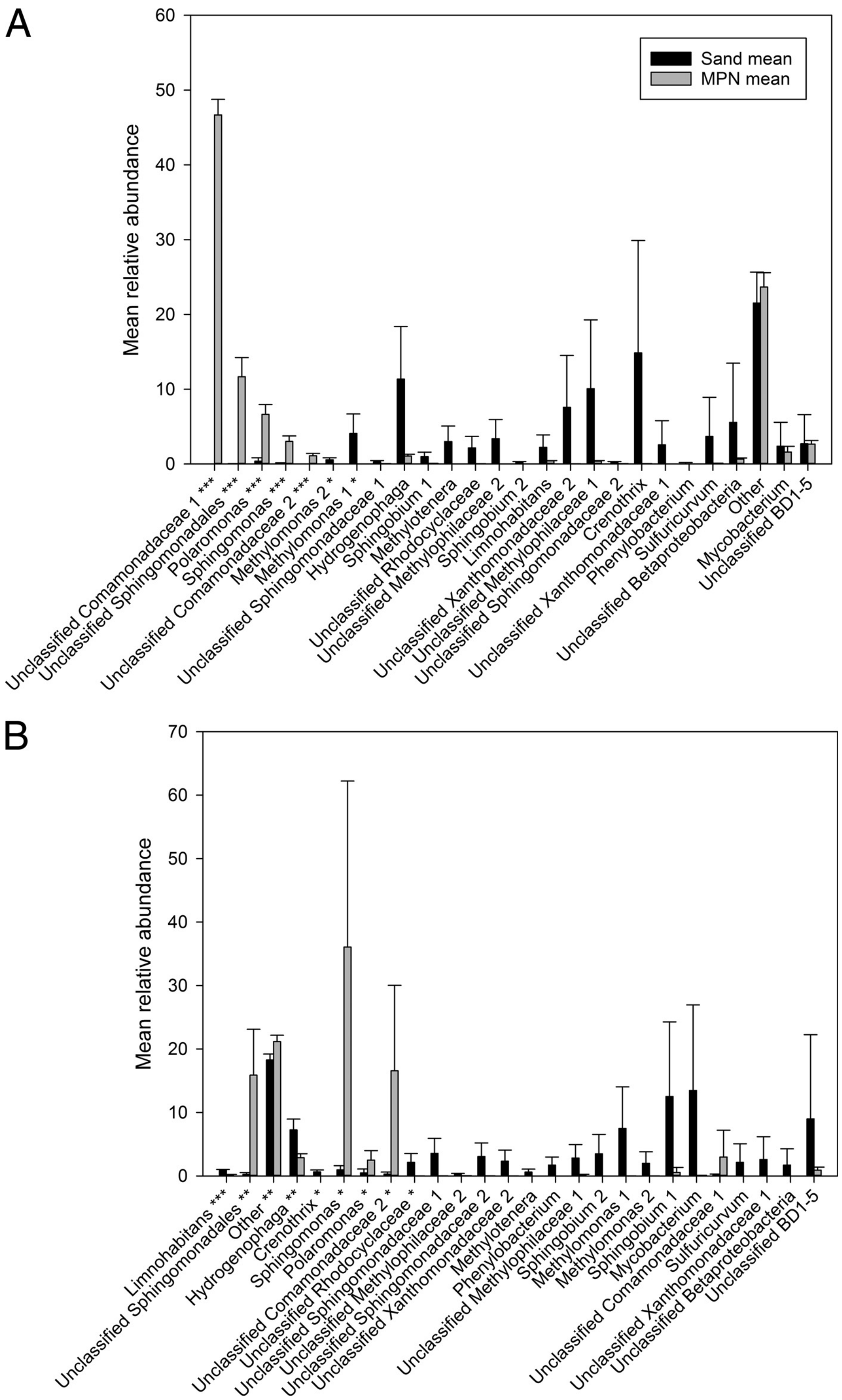

FIG 6 Relative abundance of specific OTUs in MPN and sand samples for the top of the filter (A) and the depth of the filter (B). Significant differences in mean abundance are shown with the following confidence levels: $P = 0.001$ (***), $P = 0.01$ (**), and $P = 0.05$ (*).

The *rdpA* gene was much less abundant than the *sdpA* gene in the sand filter. However, selection for bacteria harboring the *rdpA* degradation gene finally occurred, and in the top of the filter, the ratio of the *rdpA* gene copy number to bacterial number increased from <0.03% at day 14 to approximately 1% after 85 days. In the depth of the filter, the ratio was 1% at day 14 but then decreased during the study until it reached 2% at day 85.

The contaminated groundwater that was used in this study has previously been shown to contain equal concentrations of the *R* and *S* enantiomers of the phenoxypropionate herbicides (40). One could therefore expect the selection of bacteria that can use either of these enantiomers as the substrates. We showed here that the selection of both *R*- and *S*-enantiomer-specific degradation genes in fact occurred in the sand filter for 3 months but that colonization by bacteria harboring the *rdpA* gene occurred much slower than colonization by bacteria harboring the *sdpA* gene. This could be due to a lower frequency of the *rdpA* gene than the *sdpA* gene with the incoming abstracted groundwater. While isolated phenoxypropionate degraders have been shown to contain both the *sdpA* and the *rdpA* genes (24, 41), the abundance of the *rdpA* and *sdpA* genes in an unexposed agricultural soil showed a dominance of the *sdpA* gene (8). Whether *sdpA* dominance is a general phenomenon in the environment remains to be investigated, but this obviously could have an influence on the length of the lag phase that is required before effective biodegradation of the *R* enantiomer is initiated. It also means that, in fact, the 20 to 30% degradation achieved at day 85 could be a 40 to 60% degradation of the *S* enantiomer and no or minor degradation of the *R* enantiomer. Enantioselective degradation should therefore be taken into consideration in future studies on phenoxypropionate degradation in rapid sand filters.

The identity and diversity of environmental bacteria that can degrade phenoxypropionate herbicides remains unexplored to a large extent. Relatively small numbers of such bacterial isolates have been described in the literature. These included a *Rhodoferax* sp. (42) and *Delftia acidovorans* (24) isolated from herbicide-contaminated building rubble and *Sphingobium herbicidovorans* (43), an *Alcaligenes* sp. and a *Ralstonia* sp. (44), *Alcaligenes denitrificans* (45), and a number of *Sphingomonas* sp. strains (46) isolated from soil. A more comprehensive overview of degraders would be desirable, in order to get a better understanding of the degradation process and factors controlling it. Furthermore, a broader selection of degrader isolates could also potentially reveal an unknown diversity of degradation genes, which are not targeted with the PCR primers currently available. In this study, we used Illumina MiSeq sequencing of 16S rRNA genes to identify the bacteria involved in degradation in the sand filter. We used culturing in MPN plates with DCPP as the single C source to obtain an enrichment of phenoxypropionate degrader bacteria. Among all bacteria from MPN plate wells that were sequenced, a consortium of different bacteria was present, and hence, some of the enriched OTUs may simply be secondary degraders proliferating on the metabolites produced by the primary degraders. However, our finding that bacteria belonging to the *Comamonadaceae*, *Sphingomonadales*, and *Sphingomonas* were enriched matches well with previous reports on phenoxypropionate herbicide degraders. Within the family of *Comamonadaceae*, the genera *Delftia* and *Rhodoferax* have been confirmed to be degraders (23, 42). Within the order *Sphingomonadales*, degraders have also been found among the members of the genera *Sphingomonas* (46) and *Sphingobium* (43). To our knowledge, phenoxypropionate herbicide degraders have not previously been found within the genus *Polaromonas*, which was also among the OTUs enriched in the MPN plate wells. We propose that each of these bacteria play a role in degradation; however, the actual capacity as primary or secondary degraders should be further investigated.

In conclusion, this study showed a significant biodegradation of the common groundwater contaminants MCPP, DCPP, and 4-CPP, using a simple procedure, including aeration and filtration through a rapid biological sand filter. Thus, a natural microbial population of degraders existed in the environment, and within 3 months these had successfully established in the sand filter. By selective culturing and sequencing, we identified a number of bacterial representatives that benefited from phenoxypropionate degradation and thus could be future targets for isolation and further investigation.

ACKNOWLEDGMENTS

Krüger A/S is thanked for installing the pilot facility and for technical support. Specifically, Liselotte Clausen of Krüger A/S is thanked for valuable discussions on the pump-and-treat approach and the pilot facility setup.

Financial support was granted by the Danish Strategic Research Council (Miresowa Project, grant no. 2104-08-0012) and the County of Zealand (Region Sjælland OPI project, 2012). Sequencing was done at the Environmental Microbial Genomics Group, Department of Environmental Science, Aarhus University (Roskilde, Denmark), with support from the Danish Centre for Environment and Energy (DCE).

FUNDING INFORMATION

Region Sjaelland (County of Zealand) provided funding to Christian Nyrop Albers. Strategiske Forskningsråd (DSF) provided funding to Jens Aamand under grant number 2104-08-0012.

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